Electro-acupuncture exerts beneficial effects against cerebral ischemia and promotes the proliferation of neural progenitor cells in the cortical peri-infarct area through the Wnt/β-catenin signaling pathway

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Received January 23, 2015; Accepted August 21, 2015

DOI: 10.3892/ijmm.2015.2334

Abstract. Electro-acupuncture (EA) is a novel therapy based on combining traditional acupuncture with modern electrotherapy, and it is currently being investigated as a treatment for ischemic stroke. In the present study, we aimed to investigate the mechanisms through which EA regulates the proliferation of neural progenitor cells (NPCs) in the cortical peri-infarct area after stroke. The neuroprotective effects of EA on ischemic rats were evaluated by determining the neurological deficit scores and cerebral infarct volumes. The proliferation of the NPCs and the activation of the Wnt/β-catenin signaling pathway in the cortical peri-infarct area were examined. Our results revealed that EA significantly alleviated neurological deficits, reduced the infarct volume and enhanced NPC proliferation [nestin/glial fibrillary acidic protein (GFAP)-double positive] in the cortex of rats subjected to middle cerebral artery occlusion (MCAO). Moreover, the Wnt1 and β-catenin mRNA and protein levels were increased, while glycogen synthase kinase-3 (GSK3) transcription was suppressed by EA. These results suggest that the upregulatory effects of EA on the Wnt/β-catenin signaling pathway may promote NPC proliferation in the cortical peri-infarct area after stroke, consequently providing a therapeutic effect against cerebral ischemia.

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Abbreviations: EA, electro-acupuncture; NPCs, neural progenitor cells; MCAO, middle cerebral artery occlusion; SVZ, subventricular zone; SGZ, subgranular zone; TTC, 2,3,5-triphenyltetrazolium chloride; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery

Key words: electro-acupuncture, cerebral ischemia, Wnt/β-catenin, neural progenitor cells, proliferation

Introduction

Stroke is the leading cause of adult disability, as well as the second most common cause of mortality worldwide (1). Ischemic stroke accounts for approximately 80% of all strokes (1,2). Over the past decade, thrombolysis has been established as an effective treatment in the most acute phase of ischemic stroke. However, many patients develop lifelong disabilities following ischemic stroke as they do not receive the necessary treatment within the therapeutic time window. Therefore, increasing the window of therapeutic efficacy of established treatments or identifying other therapies with alternative targets is necessary to improve the neurological outcomes in ischemic stroke victims. Targeting key cellular survival/proliferation mechanisms may improve prognosis. Recently, it was reported that the signaling molecule, β-catenin, is degraded in the peri-infarct area of the brain following focal cerebral ischemia (3-7). In a previous study, it was demonstrated that in doubleridge mice, which have a reduced expression of Dkk-1, an antagonist of Wnt/β-catenin signaling, the reduction of β-catenin was attenuated and the infarct volume was reduced following middle cerebral artery occlusion (MCAO) (5). This suggests that prevenint the decrease in Wnt/β-catenin signaling in cerebral ischemia may prove to be a potential novel therapeutic modality.

In addition, the conditional expression of stabilized β-catenin in neural progenitor cells (NPCs) enlarges the cortical surface area through the expansion of the progenitor cell population (8). The overexpression of Wnt3 has been shown to increase neurogenesis in adult hippocampal precursor cells, while the blockade of Wnt signaling reduces neurogenesis both *in vitro* and *in vivo* (9). These studies also implicate the canonical Wnt/β-catenin pathway in the proliferation and self-renewal of NPCs (3,8,9).

Traditional Chinese medicine (TCM) has long been an important component of complementary and alternative medicine in

several Asian countries, and recently in Western society. Modern research has revealed the potential therapeutic effects of TCM in the treatment of various diseases, including cerebrovascular diseases and cancer (10,11). Its unique functions in gene therapy have also been discussed (12,13). Electro-acupuncture (EA) is a traditional therapeutic method used in China, widely used for both the prevention and treatment/rehabilitation of cerebral ischemia. Nevertheless, the mechanisms responsible for its effects are not yet fully understood. Previous studies have indicated that EA significantly attenuates neurological deficits, and reduces infarct volume and mortality in both animal models of stroke and in patients suffering from stroke when administered at appropriate acupoints with suitable stimulation parameters (14-19). Two specific acupoints, Quchi (LI11) and Zusanli (ST36), are one of the most effective prescriptions commonly used in EA treatment of ischemic stroke (17,20). Preliminary data have demonstrated that EA at these two acupoints significantly promotes NPC proliferation following cerebral ischemia in the subventricular zone (SVZ) of the lateral ventricle, and in the subgranular zone (SGZ) of the hippocampus (17,20). A growing body of evidence suggests that cortex-derived neural stem/progenitor cells may contribute to the repair of ischemic lesions of the cerebral cortex (21-23). Based on these and other findings, the elucidation of the Wnt signaling mechanisms underlying the promoting effects of EA on NPC proliferation in the cortical peri-infarct area after stroke, is an important step toward validating the clinical application and benefits of this treatment modality in the treatment of ischemic stroke.

Materials and methods

Materials and reagents. TRIzol reagent was purchased from Life Technologies (Carlsbad, CA, USA). The RevertAid™ First Strand cDNA Synthesis kit and Taq DNA Polymerase were purchased from Fermentas (Hanover, MD, USA). Primary antibodies against glial fibrillary acidic protein (GFAP, a marker for reactive astrocytes; #3670), glycogen synthase kinase-3 (GSK3; #5676) and β-actin (#4970), and horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse, #7076; anti-rabbit, #4970) were all obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-microtubule-associated protein 2 (MAP2, a marker of neurons; ab32454), anti-nestin (a marker of progenitor cells and astrocytes; 2Q178) and anti-β-catenin (ab22656) primary antibodies were all obtained from Abcam (Cambridge, MA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals and groups. Adult male Sprague-Dawley rats (weighing 250-280 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All experiments were performed strictly in accordance with the International Ethical Guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

A total of 72 rats were randomly divided into 4 groups (18 rats in each group) as follows: i) sham-operated (sham) group; ii) MCAO group; iii) MCAO + EA group: ischemic rats treated with EA at the Quchi (LI11) and Zusanli (ST36) acupoints; and iv) sham + EA group: sham-operated rats treated with EA.

Induction of focal cerebral ischemia. A rat model of focal cerebral ischemia/reperfusion (I/R) was utilized in this study. The left middle cerebral artery (MCA) was occluded by the placement of an embolus at the origin of the MCA, as previously described (24). Following anesthetization with 10% chloral hydrate (300 mg/kg), each rat was placed in the prone position. A midline incision was made on the dorsal surface of the skull, and the skull was thinned with a burr hole over the left parietal cortex (5 mm lateral and 1 mm posterior to the bregma) without injury to the dura mater. The laser Doppler perfusion monitor (LDF100C; Biopac Systems, Inc., Goleta, CA, USA) was attached to the skull with dental cement. With the rat in a supine position, MCAO was performed via ligation of the left common carotid artery (CCA) and external carotid artery (ECA) and closure of the internal carotid artery (ICA). The embolus was gently advanced within the left ICA to the origin of the MCA, until a slight resistance was encountered (20±2 mm). Cerebral blood flow was measured beginning 5 min prior to the induction of occlusion. Ischemic rats that showed a stable drop of 80% in blood perfusion units (BPU) compared with the baseline level (before MCAO), were used in the subsequent experiments. Reperfusion was achieved by removing the intraluminal occlusive embolus to restore blood supply to the MCA area 2 h later. Animals subjected to sham operation were treated in a similar manner, but without ligations and occlusions.

Neurological assessment. Neurological deficits were assessed to confirm successful MCAO. A neurological score was assigned to each animal 2 h following I/R, in a blinded manner, according to a well-established 5-point neurological scale (24): score 0, no apparent deficits; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling or leaning over to the right; 4, no spontaneous walking and a depressed level of consciousness; and 5, dead. Rats subjected to MCAO with neurological deficit scores of 1-3 were used in the subsequent experiments.

Treatment wtih EA. EA was applied at the LI11 (Quchi, in the depression lateral to the anterior aspect of the radius joint of the forelimb) and ST36 (Zusanli, 5 mm below the head of fibula under the knee joint and 2 mm lateral to the anterior tibial tubercle) acupoints on the right paralyzed limb using an EA stimulation instrument [Model G6805; Shanghai Marine Instrument General Factory (SMIF), Shanghai, China]. Two stainless steel acupuncture needles, 0.3 mm in diameter, connected to the output terminals of the EA stimulation instrument, were inserted at a depth of 2-3 mm at the LI11 and ST36 acupoints. The acupoints were stimulated with disperse-dense waves of 1 or 20 Hz frequencies for 30 min, once a day, and the current intensity was maintained slightly below the level that induced visible muscle contraction. Treatment commenced on the day following the operation and continued daily until the animals were sacrificed.

Measurement of cerebral infarct volume. Three days following cerebral I/R injury, the rats were euthanized under deep anesthesia using 10% chloral hydrate and perfused transcardiacally with 0.9% NaCl. The brains of all the rats were rapidly removed and sliced into 5 coronal blocks at a thickness of 2 mm per section. The fresh slices were incubated in 2% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich)

Table I. Primer sequences used for PCR.

Gene names	Forward	Reverse
Wnt1	5'-CAG TGG AGC AAC GGT ATG AG-3'	5'-TTC TTC CCT GCC TTG ATG T-3'
GSK3	5'-AGA CCA AAA TCA TCT ACC AC-3'	5'-ACT CTG TGC CTG TCT CAT-3
β-catenin	5'-CAT CCT TAT CCC TCC TCA CGC-3'	5'-TTA TTG GTC TGT CCA CGG TCT-3''
β-actin	5'-CGG GAG AAC AGG GTA TGA-3'	5'-CAG GCT GGA AGG AGA AGA T-3'

GSK3, glycogen synthase kinase-3.

solution in phosphate-buffered saline (PBS; HyClone, Beijing, China) for 20 min, at 37˚C in the dark. Images of the 5 sections were captured using a high-resolution digital camera (PowerShot SX20 IS; Canon) and examined by a blinded observer to determine the infarct size using computerized image analysis software (Motic Med 6.0 system; Motic China Group Co.,. Ltd., Shenzhen, China). The infarct volume data are expressed as a percentage of the total brain volume. Lesion volume was estimated using an indirect method to avoid the effects of tissue swelling or shrinkage: 100x (contralateral hemisphere volume - non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume, as previously described (25).

Tissue preparation. The rats were anesthetized with 10% chloral hydrate and intracardially perfused with chilled saline followed by 0.01 M PBS containing 4% paraformaldehyde through the left ventricular lumen of the heart. The brains were collected and post-fixed in 4% paraformaldehyde at 4˚C overnight, and then embedded in paraffin. Coronal sections were cut into 5-μm-thick sections, and used for immunofluoresence staining. For western blot analysis RT-PCR, the ischemic boundary zones were extracted from the ischemic brains and prepared accordingly.

Immunofluorescence staining. The brain sections were processed by immunofluorescence staining using several specific cell markers (MAP2, neurons; GFAP, astrocytes; nestin, NPC and astrocytes). Coronal sections (5-μm-thick) were de-paraffinized in dimethylbenzene, hydrated successively in gradient ethanols, and antigens were retrieved twice in 0.1 M citrate buffer (pH 6.0). The sections were blocked in blocking buffer (10% normal goat serum, 0.3% Triton X in PBS) for 1 h at room temperature, then incubated with primary antibodies at 4˚C overnight. After washing in 0.01 M PBS, the sections were incubated for 2 h at room temperature with a combination of goat anti-mouse IgG H&L (FITC; ab6785; Abcam) and goat anti-rabbit IgG H&L (TRITC; ab6718; Abcam) secondary antibodies. The sections were stained with DAPI (Vector Laboratories, Burlingame, CA, USA) to localize the nuclei and were coverslipped for observation. The labelled sections were visualized and imaged using a confocal microscope (LSM710 META NLO; Carl Zeiss, Oberkochen, Germany).

Western blot analysis. The left cerebral tissues were dissected out and homogenized in RIPA buffer containing Protease Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany) and PMSF. The samples were kept on ice for 30 min and the insoluble material was removed by centrifugation at 14,000 x g for 15 min. The protein concentration was quantified by BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Brain homogenates (50 μg) were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were subsequently blocked for 2 h with 5% non-fat powdered milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated overnight at 4˚C with appropriate primary antibodies: GFAP, Wnt1 (SAB2102711; Sigma-Aldrich), GSK3, β-catenin and β-actin (at a dilution of 1:1,000). The membranes were then washed with TBST followed by incubation with the appropriate HRP-conjugated secondary antibody for 1-2 h at room temperature. Normalization of the results was ensured by running parallel western blot analyses with β-actin antibody. The optical density was quantified using a Bio-Image Analysis System (Bio-Rad, Hercules, CA, USA), with the value of the sham-operated group designated as 1.0.

RNA extraction and RT-PCR. Total RNA was extracted using TRIzol Reagent (Life Technologies). The RNA concentrations were determined by OD260/280 readings using a GeneQuant spectrophotometer (Amersham Biosciences, Amersham, UK). The oligo(dT)-primed RNA (3 μg) was reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Chicago, IL, USA) according to the manufacturer's instructions. Semi-quantitative PCR was performed to measure the Wnt1, GSK3, β-catenin and β-actin mRNA expression levels. The primer sequences used for each gene are listed in Table I. The samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation System (Model Gel Doc 2000; Bio-Rad), with the value of the sham-operated group designated as 1.0.

Cell quantification and statistical analysis. The infarct area was defined by tissue autofluorescence, while the peri-infarct area was defined by the presence of MAP2-positive immunofluorescent cells. Cell quantification in the cortical peri-infarct area was performed by observers blinded to the sample identity using Image-Pro Plus 6.0 software. The results are expressed as the number of MAP2-positive cells/cm^2. All data were analyzed using the SPSS package for Windows (version 16.0) and are presented as the means ± standard error of the mean (SEM). Statistical data analysis was performed with the unpaired Student's t-test, the Mann-Whitney U test

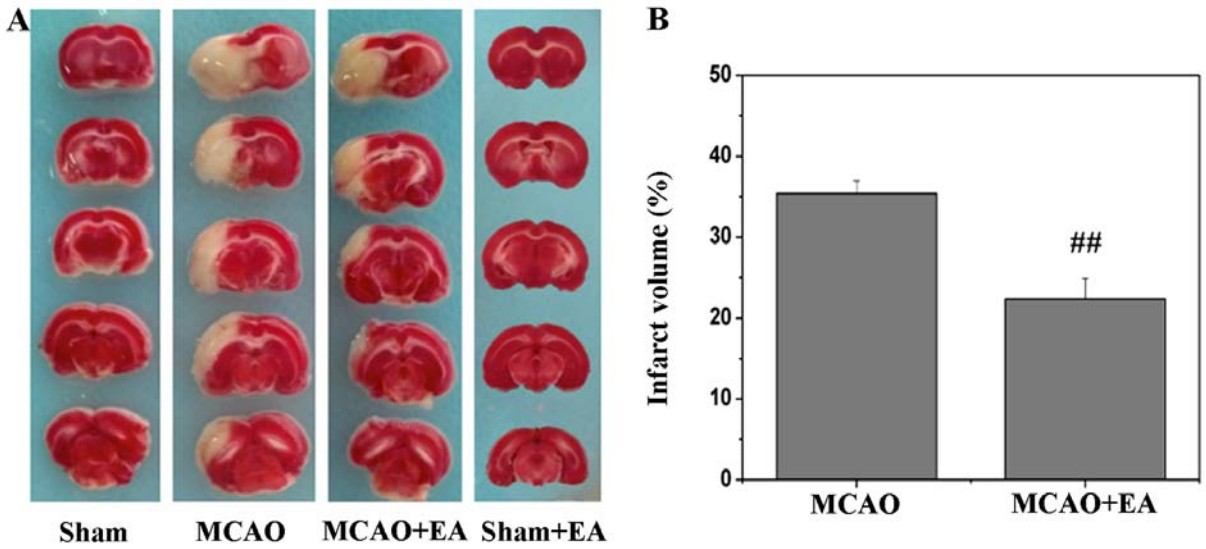

Figure 1. Effects of electro-acupuncture (EA) on cerebral infarction after stroke. (A) Cerebral tissues from each group were coronally sectioned into 2-mm-thick slices and then processed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. Normal areas of the brain were stained deep red, indicating intact mitochondrial function, whereas the infarct areas remained unstained. Representative images were captured using a high-resolution digital camera. (B) Infarct volume was quantified using the Motic Med 6.0 system, and is presented as a percentage of the total brain volume. Data shown are the means ± SEM from 5 individual rats in each group. $^{\#\#}$P<0.01 vs. MCAO group. Sham, sham operation, control; MCAO, middle cerebral artery occlusion.

Table II. Assessment of neurological deficits.

Group	2 h after I/R	3 days after I/R
Sham	0	0
MCAO	2.33±0.19	2.08±0.23
MCAO + EA	2.42±0.19	1.42±0.19[a]
Sham + EA	0	0

Data shown are the means ± SEM from 18 individual rats in each group. [a]P<0.05 vs. the MCAO group. Sham, sham operation, controls; MCAO, middle cerebral artery occlusion; EA, electro-acupuncture; I/R, ischemia/reperfusion; SEM, standard error of the mean.

or ANOVA. Differences with P<0.05 were considered statistically significant.

Results

EA alleviates neurological deficits and reduces infarct volume after stroke. Compared with the rats in the sham-operated and sham + EA groups, which did not present with any signs of cerebral injury, all rats in both the MCAO and MCAO + EA groups demonstrated obvious manifestations of neurological deficits and cerebral infarction (Fig. 1 and Table II). There were no statistically significant differences observed between the MCAO and MCAO + EA groups at 2 h after cerebral I/R injury. However, EA administered at the Zusanli and Quchi acupoints for 3 days significantly improved neurological deficits (MCAO, 2.08±0.23; MCAO + EA, 1.42±0.19; P<0.05) (Table II), and decreased the cerebral infarct volume (MCAO, 35.39±1.56%; MCAO + EA, 22.39±2.50%; P<0.01) (Fig. 1), demonstrating the therapeutic efficacy of EA against cerebral I/R injury.

EA accelerates the proliferation of GFAP-positive reactive astrocytes in the cortical peri-infarct area after stroke. The border of the infarct core was defined by MAP2 staining (Fig. 2), and the subsequent results identified a few GFAP-positive cells in the non-ischemic cortex (Fig. 3E and H); however, the majority of GFAP-positive cells were observed in the post-stroke cortex, specifically in the peri-infarct area (Fig. 3F and G). Western blot analysis also revealed a significant increase in GFAP expression in the MCAO and MCAO + EA groups within the post-stroke cortex, compared with the comparable MCA area in the sham-operated (sham) and sham + EA groups (sham, 1±0; MCAO, 1.95±0.24; MCAO + EA, 3.10±0.44; and sham + EA, 0.97±0.07; P<0.01 vs.sham and sham + EA groups; Fig. 3I and J). In addition, overall GFAP expression was significantly higher in the MCAO + EA group than in the MCAO group (P<0.05), suggesting that treatment with EA promoted the proliferation of GFAP-positive reactive astrocytes in the cortical peri-infarct area after stroke.

EA enhances the proliferation of NPCs in the cortical peri-infarct area after stroke. The presence of nestin/GFAP-positive cells within the post-stroke cortex was investigated in order to assess the generation of injury-induced NPCs. Previous studies have reported that nestin and GFAP-positive cells can acquire stem cell activity in the cortical peri-infarct area after stroke (21,22). In the present study, a nestin-positive subpopulation of NPCs formed on the ipsilateral, but not the contralateral side of the brain after stroke. At 3 days after stroke, the number of nestin-positive cells was significantly higher in the MCAO + EA group than in the MCAO group. Similarly, the number of nestin/GFAP-positive cells was significantly higher in the MCAO + EA group (MCAO, 257.72±49.73; MCAO + EA, 379.56±20.05; P<0.05; Fig. 4), demonstrating that EA potentially increased the proliferation of the NPCs in the cortical peri-infarct area after stroke.

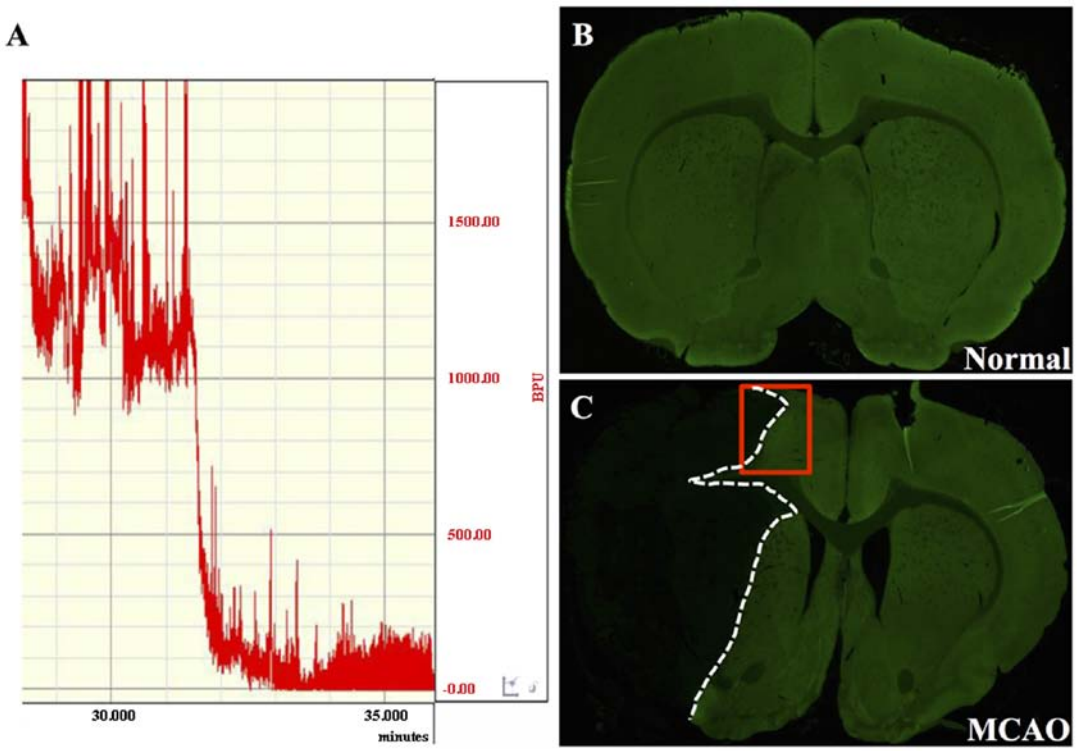

Figure 2. Changes in cerebral blood flow in response to middle cerebral artery occlusion (MCAO). Trace recordings of the blood flow in the ischemic brains were measured using a laser Doppler perfusion monitor. (A) MCAO immediately decreased the perfusion units (PU) to 20% of the base value, was regarded as a successful model. Tissue corresponding to (B) normal and the (C) peri-infarct area. The area peri-infarct is depicted by the red rectangle. The border of the infarct core (dotted line) was defined by microtubule-associated protein 2 (MAP2) staining.

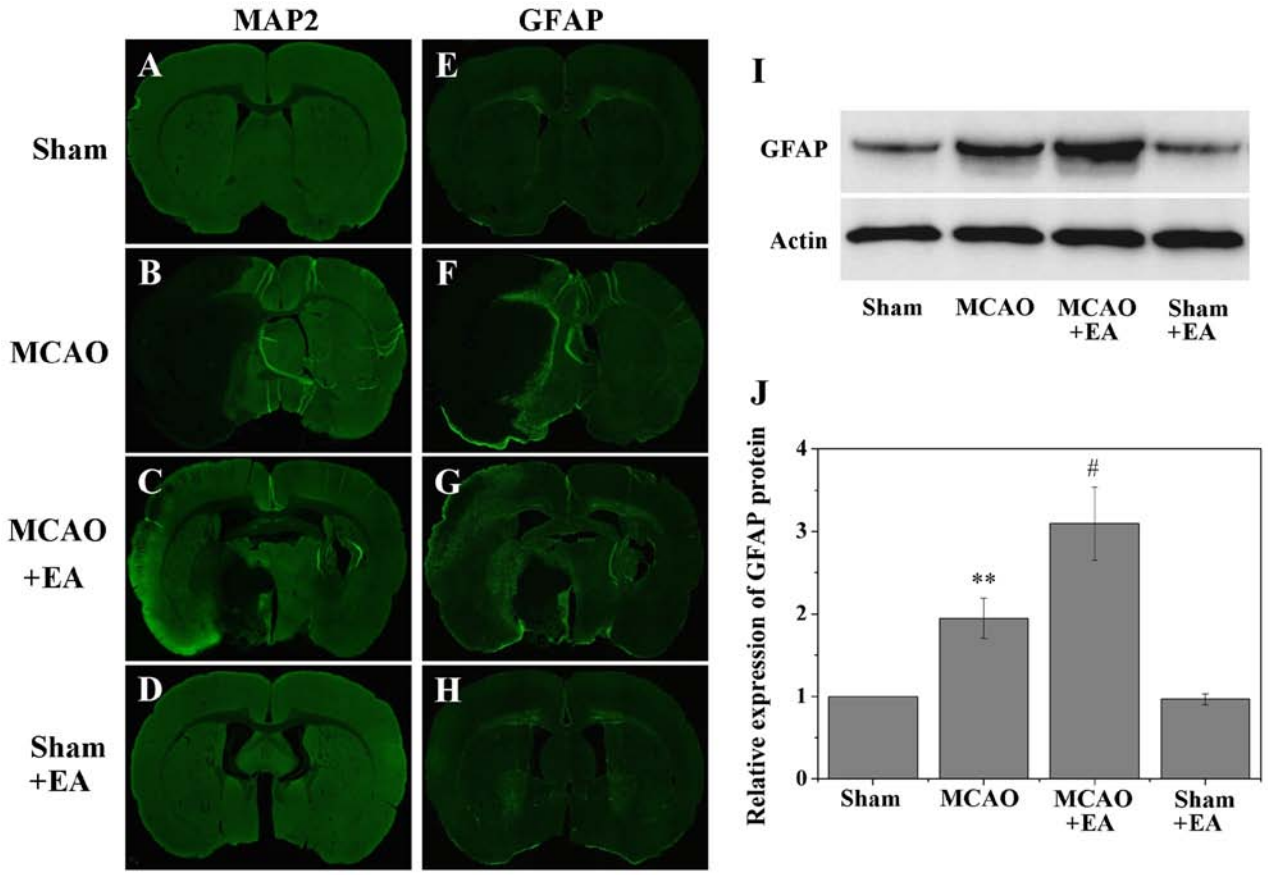

Figure 3. Effect of electro-acupuncture (EA) on glial fibrillary acidic protein (GFAP)-positive reactive astrocytes after stroke. (A-D) The border of the infarct core is defined by microtubule-associated protein 2 (MAP2) staining (green). (E-H) Cells expressing GFAP were identified by immunofluorescence staining (green) and (I and J) western blot analysis. Data are representative of 5 individual rats from each group. **P<0.01 vs. sham (sham-operated) and sham + EA groups; $^{\#}$P<0.05 vs. middle cerebral artery occlusion (MCAO) group.

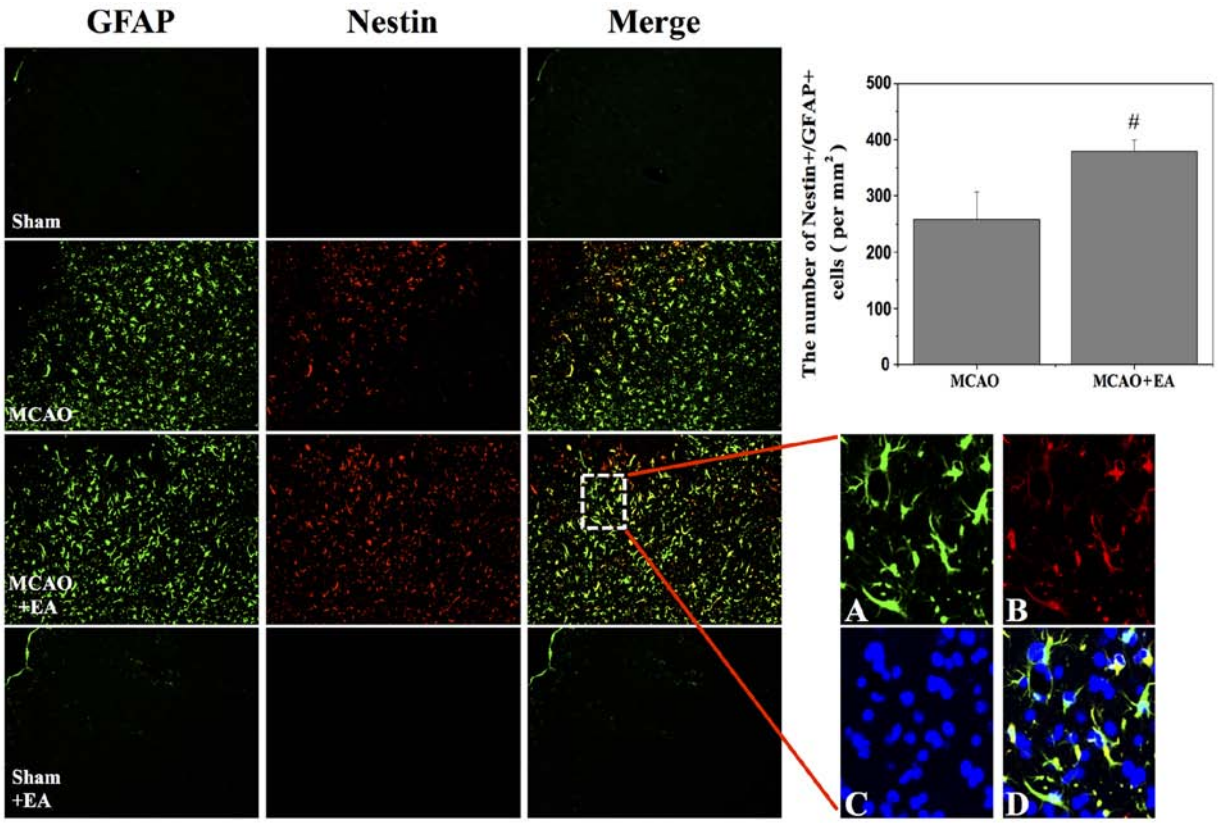

Figure 4. Effects of electro-acupuncture (EA) on neural progenitor cell (NPC) proliferation after stroke. Representative double immunofluorescence staining for glial fibrillary acidic protein (GFAP) (green) and nestin (red) in the peri-infarct area. (A-D) Higher magnification of insets are indicated by white squares. Nuclei were counterstained with DAPI (blue). Data shown as the means ± SEM from 5 individual rats from each group. $^{\#}$P<0.05 vs. middle cerebral artery occlusion (MCAO) group.

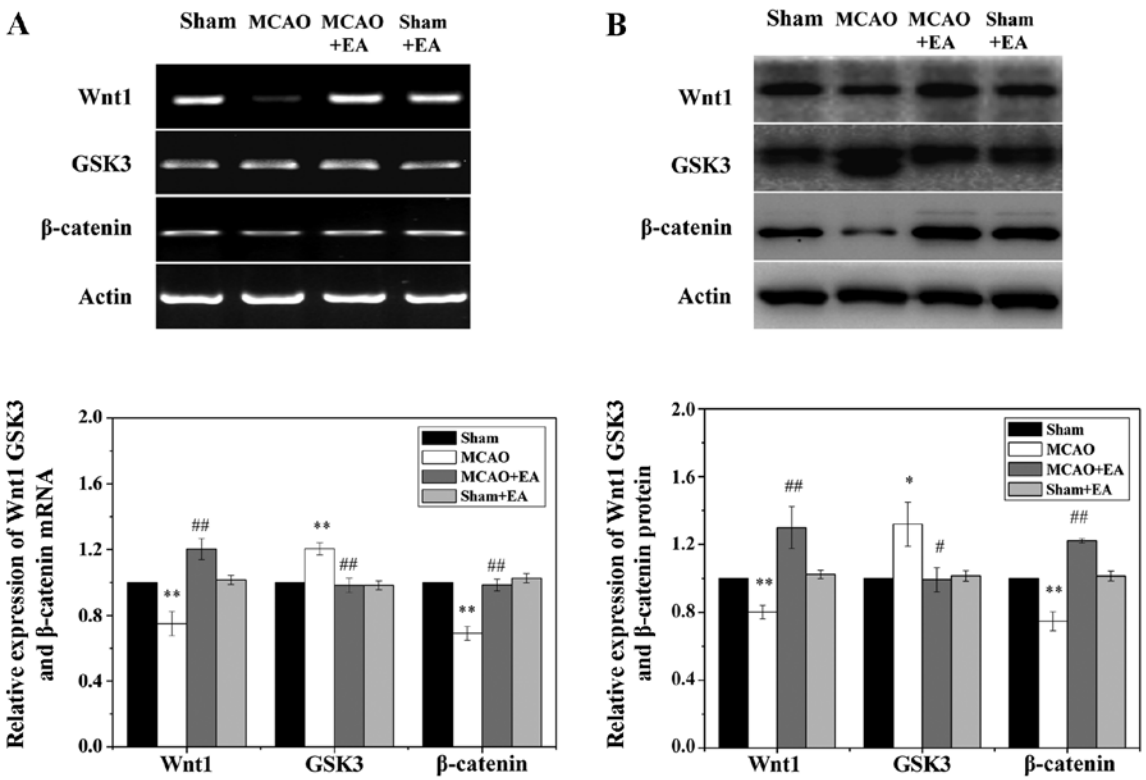

Figure 5. Effects of electro-acupuncture (EA) on the activation of the Wnt signaling pathway. (A) The mRNA levels of Wnt1, glycogen synthase kinase-3 (GSK3) and β-catenin in the cortical peri-infarct area were measured by RT-PCR. (B) The protein expression levels of Wnt1, GSK3 and β-catenin were measured by western blot analysis. β-actin was used as an internal control for both RT-PCR and western blot analysis. Data are representative of 4 individual rats from each group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. middle cerebral artery occlusion (MCAO) group; *P<0.05, **P<0.01 vs. sham and sham + EA groups.

EA regulates the activation of the Wnt pathway in the cortical peri-infarct area after stroke. To examine the effects of EA on the Wnt signaling pathway, we measured the protein and mRNA levels of Wnt1, GSK3 and β-catenin in the ischemic cortex by western blot analysis and RT-PCR. As shown in Fig. 5, focal cerebral I/R injury significantly reduced the expression of Wnt1 and β-catenin, while the transcription of GSK3 was significantly increased in the ischemic cortex at 3 days following stroke when compared to the sham and sham + EA groups ($P<0.05$). Of note, the decrease in the expression of Wnt1 and β-catenin was circumvented by treatment with EA, and the upregulated transcription of GSK3 was significantly reduced following treatment with EA ($P<0.05$). Taken together, these results suggest that EA applied at the Quchi and Zusanli acupoints significantly promotes the activation of the Wnt signaling pathway in the peri-infarct cortex.

Discussion

In response to stroke, subpopulations of cortical reactive astrocytes proliferate and express several proteins commonly associated with neural stem/progenitor cells, such as GFAP, nestin and RC2 (21-23). Shimada *et al* (21) demonstrated that GFAP-expressing reactive astrocytes can be isolated from the cortical peri-infarct area 3 days after stroke, and de-differentiated into reactive astrocyte-derived neural stem/progenitor cells with self-renewal and multipotent properties when grown under neurosphere conditions. Lineage tracing identified reactive astrocytes as a cell of origin for neural stem cells (NSCs) derived from cortical peri-infarct tissues after stroke (21).

In this study, in order to investigate the effects of EA on NPC proliferation via Wnt signaling, we treated sham-operated and rats subjected to MCAO with electric stimulation at the Quchi (LI11) and Zusanli (ST36) acupoints on the contralateral paralyzed limb. Our results revealed that EA applied at these acupoints 1 day following cerebral I/R injury and once daily for 3 consecutive days, significantly improved neurological function (MCAO, 2.08±0.23; MCAO + EA, 1.42±0.19; $P<0.05$) and attenuated the increase in the cerebral infarct volume (MCAO, 35.39±1.56%; MCAO + EA, 22.39±2.50%; $P<0.01$) induced by MCAO. Our findings corroborate those of previous studies that used a model of transient focal cerebral ischemia to demonstrate the therapeutic efficacy of EA (14-17). Furthermore, immunofluorescence staining was performed to observe several markers associated with the activation of NPCs. Our results revealed that the number of proliferating nestin/GFAP-positive NPCs was significantly increased within the post-stroke cortex in the MCAO group (257.72±49.73) (Fig. 4) but even more so in the MCAO + EA group (379.56±20.05, $P<0.05$ vs. MCAO group), suggesting that EA promoted the proliferation of neural stem/progenitor cells in rats subjected to MCAO. The emergence of NPCs in the peri-infarct area is a documented event in the brain after stroke (21-23). However, the signaling pathway controlling the proliferation of these NPCs is poorly defined.

Of note, previous studies have indicated that Wnt/β-catenin signaling is critically involved in the regulation of the proliferation and differentiation of NPCs (3,8,9). The Wnt/β-catenin pathway is activated when a Wnt ligand binds to its seven-transmembrane receptors, the Frizzled proteins. The activation of the Wnt pathway inhibits GSK-3β, which results in the cytoplasmic accumulation of β-catenin. Stabilized β-catenin then translocates into the nucleus and interacts with the transcription factors TCF/Lef to activate downstream genes such as cyclin D1 and c-myc (26,27).

Cerebral ischemia profoundly reduced the transcription of Wnt1 and β-catenin and increased the expression of GSK3. Treatment with EA reversed these effects (Fig. 5). Moreover, this is consistent with the results of previous reports that the Wnt pathway is markedly degraded after stroke (3-5). In a previous study, when assessed at 3 days following an endothelin-1 (Et-1) injection, treatment with lithium ions prevented the decrease in the expression of β-catenin in the ischemic cortex (5). In this study, the treatment of NPCs with EA significantly increased the expression of Wnt1 and β-catenin, while inhibiting the transcription of GSK3. These data indicate that EA applied at the Quchi (LI11) and Zusanli (ST36) acupoints promoted the proliferation of NPCs in the cortical peri-infarct area via the Wnt/β-catenin pathway.

In conclusion, the results of the present study strongly suggest that treatment with EA provides robust protection against transient cerebral ischemic injury and promotes the proliferation of neural stem/progenitor cells in response to ischemia via the Wnt/β-catenin pathway. Our data are supported by evidence in the current literature. These results may provide a theoretical and experimental basis for the future clinical application of EA and its potential use in the treatment of cerebral ischemia.

However, even with optimal stimulation parameters, treatment with EA targets multiple mechanisms in order to achieve its protective effects against ischemic insults. Therefore, the precise mechanisms of action associated with this treatment the reparative process in the post-ischemic brain requires further investigation.

Acknowledgements

This study was sponsored by the National Natural Science Foundation of China (grant nos. 81273835 and 81373778). We would like to thank Clarity Manuscript Consultants, LLC, for their assistance in the editing of this manuscript.

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